



Investigation of the insulin-like growth factor system in the avian epiphyseal growth plate

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Abstract

Components of the insulin-like growth factor (IGF) system were investigated in chondrocytes isolated from the avian growth plate. The genes for IGF-I, IGF-II, type 1 IGF receptor (IGF-R), IGF binding protein-2 (IGFBP-2), IGFBP-3, IGFBP-5 and IGFBP-7 were found to be expressed in both proliferative and hypertrophic chondrocytes. The expression of IGF-II in proliferative chondrocytes was extremely high relative to IGF-I. Although IGF-I expression was significantly increased in hypertrophic chondrocytes, the level was still low relative to IGF-II. In cell culture, IGF-I stimulated proteoglycan synthesis and increased the expression of Indian hedgehog (Ihh) and type X collagen, markers of chondrocyte differentiation. IGF-II was found to be equally efficacious in stimulating proteoglycan biosynthesis. These observations suggest that IGF-II may play a significant role in avian growth plate physiology, which is consistent with several reports on mammalian endochondral bone growth. © 2006 Elsevier Inc. All rights reserved.

Keywords: IGFs; Gene expression; Insulin-like growth factor system; Epiphyseal growth plate; Chicken

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1. Introduction

The epiphyseal growth plate is the tissue responsible for longitudinal skeletal growth. Regulation of activity of the growth plate is under the control of systemic hormones such as growth hormone (GH) and locally produced peptide growth factors. Insulin-like growth factors (IGFs) have long been thought to influence the metabolism of growth plate chondrocytes. This dates to the classic somatomedin hypothesis of Salmon and Daughaday [1] which postulates that the effects of GH are mediated through somatomedins A and C (IGF-I and IGF-II).

Since this initial observation, there have been a wide variety of studies utilizing gene mutations in the mouse to delineate the respective roles of GH and IGF-I in epiphyseal growth plate tissue. The preponderance of evidence suggests that GH has both IGF-I-dependent and independent roles in promoting mammalian chondrocyte metabolism [2,3]. Furthermore, the discovery that IGF-I is produced in this tissue suggests that this peptide can play an autocrine/paracrine role in this tissue [4].

Although considered to be important in embryonic skeletal growth [5], IGF-II also has the potential to influence post-natal skeletal metabolism. There is more IGF-II than IGF-I in extracts of vertebrate bone [6]. Since these extracts were from intact bone, the tissue which was the source of these peptides was not identified. However, Shinar et al. [7] provide better insight into the differential expression of IGF-I and IGF-II in rat skeletal tissue. Their results suggested that IGF-II plays a major role in early post-natal development of bone and longitudinal growth of the epiphyseal growth plate. IGF-I was more closely associated with the osteogenic regions of bone and did not replace the declining levels of IGF-II associated with age. These observations were confirmed by Wang et al. [8] who demonstrated cell specific patterns for the expression of IGF's receptors and IGF-binding proteins in mouse skeletal tissue.

Much less is known about the role of IGFs in avian skeletal metabolism. Several immunohistochemical studies have demonstrated the presence of IGF in embryonic limb chondrocytes [9] and post-natal growth plate tissue [10–12]. Rosselot et al. [13] demonstrated the presence of IGF-I mRNA in post-natal growth plate tissue. However, IGF-I gene expression was not correlated with the level of circulating growth hormone.

There is not total agreement on the role of IGFs in growth plate physiology. While there are reports that IGF stimulates chondrocyte proliferation [14,15], much of the evidence suggests a role in matrix synthesis and chondrocyte differentiation. For example, the original assay for somatomedin was based upon its ability to stimulate cartilage proteoglycan biosynthesis. This was confirmed with avian growth plate chondrocytes by Rosselot et al. [16]. IGF was the most potent peptide growth factor for stimulating chondroitin sulfate synthesis while being a poor mitogen for chondrocytes cultured under serum-free conditions.

The epiphyseal growth plate is organized in horizontal zones based upon the stage of differentiation of the chondrocytes. In the proliferative zone, the chondrocytes proliferate and synthesize type II collagen. These cells start to differentiate in the pre-hypertrophic zone and complete differentiation in the hypertrophic zone. In the latter stages of hypertrophy, the tissue mineralizes, is vascularized and replaced by trabecular bone. Changes in the expression of a wide variety of genes are involved in the process. Two examples are *Ihh* and type X collagen. *Ihh* is a member of the hedgehog family of intercellular signaling

proteins involved in growth and development of a wide variety of tissues [17]. It plays an important role in endochondral bone formation by coordinating with parathyroid hormone related protein (PTHrP) to regulate chondrocyte differentiation. In situ hybridization shows this signaling protein to have its highest expression in the pre-hypertrophic chondrocytes of the avian growth plate [18]. Type X collagen is a short chain collagen synthesized and secreted by hypertrophic chondrocytes [19,20]. This is used extensively as a marker for chondrocyte differentiation and hypertrophy.

Since little is known about the relative expression and bioactivity of IGF system genes in the avian epiphyseal growth plate, these studies were initiated to more clearly define the role of these factors in growth plate physiology.

2. Materials and methods

2.1. Animals and cell culture

Peterson × Arbor Acre broiler chicks were raised at The Pennsylvania State University Poultry Education and Research Center. The animals were sacrificed at 3–4 weeks of age by cervical dislocation and tibiae removed using a protocol approved by IACUC #00R158. After removing the articular cartilage, the top 10–15 cell layers of the proliferative zone were removed and minced in Ham's F12 medium. Chondrocytes were released by enzymatic digestion and plated at a density of 12.2×10^5 cells/cm³ and maintained in modified serum-free modified Webbers media (MWM) for up to 72 h. Proliferation and differentiation of the cells could be controlled by the composition of the peptide growth factor cocktail [21,22].

2.2. Proteoglycan synthesis

The incorporation of ³⁵SO₄ into cultured proliferative chondrocytes was used to measure proteoglycan synthesis. The procedure has been previously described by Rosselot et al. [16]. Briefly, proliferative chondrocytes were cultured in MWM with recombinant human IGF-I (Bachem Bioscience, Inc., King of Prussia, PA) or chicken IGF-II (GroPep, Adelaide, Australia) for approximately 48 h. During the final 12 h of incubation, the cells were pulse labeled with Na₂ ³⁵SO₄ (ICN Biomedical, Irvine, CA) (final activity, 5 μCi/ml). Nonradioactive sulfate (to minimize the effect of unincorporated radiosulfate) and trichloroacetic acid (final concentrations, 0.01 and 5%, respectively) were added before cell harvesting. The cells were filtered onto glass microfiber disks, which were then air-dried, added to scintillation fluid and counted in a liquid scintillation counter.

2.3. Northern blotting

Standard Northern blotting protocols [23] were used to determine the expression levels of *Ihh*, collagen II, collagen X and 18S. Isolated proliferative chondrocytes were cultured in serum-free MWM with IGF-I (Bachem) or recombinant human IGF-II (Intergen, Norcross, GA) for varying periods of time. Total RNA was isolated from the cultured cells using

TRIzol[®] reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer's instructions. Ten micrograms of total RNA per lane were electrophoresed through a 1% denaturing agarose gel, blotted onto a Zeta-Probe GT Genomic membrane (Bio-Rad, Hercules, CA), and then sequentially probed with ³²P-labeled cDNAs. A Molecular Dynamics Storm 860 phosphorimager and Image Quant software (Amersham Pharmacia Biotech) were used to visualize the results. The chicken *Ihh* cDNA [18] was provided by Dr. Clifford J. Tabin (Harvard Medical School, Boston, MA). The chicken collagen II [24] and collagen X [25] cDNAs were provided by Dr. Phoebe Leboy (University of Pennsylvania, School of Dental Medicine, Philadelphia, PA). Expression levels were adjusted for variability in loading using 18S rRNA, and triplicate experiments were conducted for each gene analyzed.

2.4. Reverse transcription polymerase chain reaction (RT-PCR)

Freshly isolated chondrocytes were used as a source of mRNA for this study. Proliferative cells were isolated as described for the cell culture experiments. The embryonic cone from day-old chicks was obtained immediately after hatching and served as a source of hypertrophic chondrocytes. This tissue contains a large hypertrophic zone which is vascularized and forms trabecular bone 48–72 h after hatching. Immediately after isolation, the cells were frozen and stored at –80 °C until used for mRNA isolation. Total RNA was isolated from chondrocyte samples using Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription (RT) reactions (20 µl) consisted of 1.0 µg total RNA, 50 units Superscript III reverse transcriptase (Invitrogen), 40 units of an RNase inhibitor (Invitrogen), 0.5 mM dNTPs and 100 ng of random hexamer primers. Polymerase chain reaction (PCR) was performed in 25 µl reactions containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.0 unit of Platinum Taq DNA polymerase (Invitrogen), 0.2 mM dNTPs, 2.0 mM Mg²⁺, 10 pmol of each gene specific primer, 5 pmol each of an appropriate mixture of primers:competimers specific for 18S rRNA (QuantumRNA Universal 18S Standards kit, Ambion, Inc., Austin, TX) and 1 µl of the RT reaction. Thermal cycling parameters were 1 cycle 94 °C for 2 min, followed by 30 cycles, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min with a final extension at 72 °C for 8 min. Negative RT controls were run to ensure PCR accuracy and specificity. The sequences of the primers used are presented in Table 1.

2.5. Capillary electrophoresis with laser induced fluorescence detection (CE-LIF)

Separation and quantitation of PCR products was accomplished using CE-LIF as described previously [26]. Aliquots (2 µl) of RT-PCR samples were first diluted 1:100 with deionized water. A P/ACE MDQ (Beckman Coulter, Inc., Fullerton, CA) equipped with an argon ion LIF detector was used. Capillaries were 75 µm i.d. × 32 cm µSIL-DNA (Agilent Technologies, Palo Alto, CA). EnhanceCE[™] dye (Beckman Coulter, Inc.) was added to the DNA separation buffer (Sigma, St. Louis, MO) to a final concentration of 0.5 µg/ml. Samples were loaded by electrokinetic injection at 3.5 kV for 5 s and run in reverse polarity at 8.1 kV for 3.5 min. P/ACE MDQ software (Beckman Coulter, Inc.) was used to calculate peak areas for the PCR amplicons separated by CE.

Table 1
Primers used for analysis of chondrocyte gene expression by RT-PCR

Gene	Orientation	Primer sequence (5' → 3')	Product size (bp)
1. IGF-1	Forward	GCTGAGCTGGTTGATGCTCT	202
	Reverse	CACGTACAGAGCGTGCAGAT	
2. IGF-2	Forward	ACACTGCAGTTCGTCTGTGG	400
	Reverse	GCTGACTGGGCAAGGAGAT	
3. IGF receptor (type 1)	Forward	CAAGCATGCGTGAGAGGATA	404
	Reverse	CAAACCTTTCCTCCTTTCC	
4. IGF binding protein-2	Forward	AACAGGCATGAAGGAGATGC	400
	Reverse	CTGCTCATGGGCTGTGTAGA	
5. IGF binding protein-3	Forward	CTCTTCGGAATCCAAGCAAG	400
	Reverse	CCACCACAGGAGACAGACAG	
6. IGF binding protein-5	Forward	CCTCTCTGACCTCAAAGCTGA	198
	Reverse	ACCATCCTCTGGCTGCTCT	
7. IGF binding protein-7	Forward	CCAGAGAATGGAGCTTCTGC	243
	Reverse	CATCACAGCTCTGCACCATC	

2.6. Relative quantitation of gene expression by CE-LIF

The level of gene expression by CE-LIF was determined as the ratio of the integrated peak area for each PCR product relative to that of the co-amplified 18S rRNA internal standard. Values are presented as the means \pm S.E.M.

2.7. Statistical analyses

Proteoglycan synthesis and gene expression data were subjected to analysis of variance (ANOVA) using the general linear models (GLM) procedure of SAS software (The SAS System for Windows, Version 8.2; SAS Institute, Cary, NC). The *t*-test and the Student–Newman–Keuls multiple range test options of the GLM procedure for SAS were used to determine significance of mean differences. Significance was set at $P < 0.05$.

3. Results

3.1. IGF system gene expression in freshly isolated chondrocytes

RT-PCR was used to study the expression of IGF system gene mRNAs isolated from growth plate chondrocytes. The results are shown in Fig. 1. Most of these genes were found to be expressed in both proliferative and hypertrophic chondrocytes. However, we did not detect any measurable expression of IGFBP-1 or IGFBP-4 in proliferative or hypertrophic chondrocytes (data not shown). Expression levels for IGF-II, IGF type 1 receptor and IGFBP-2 were similar for proliferative and hypertrophic chondrocytes. Of particular note is the high level of expression of IGF-II relative to IGF-I. Although the expression level

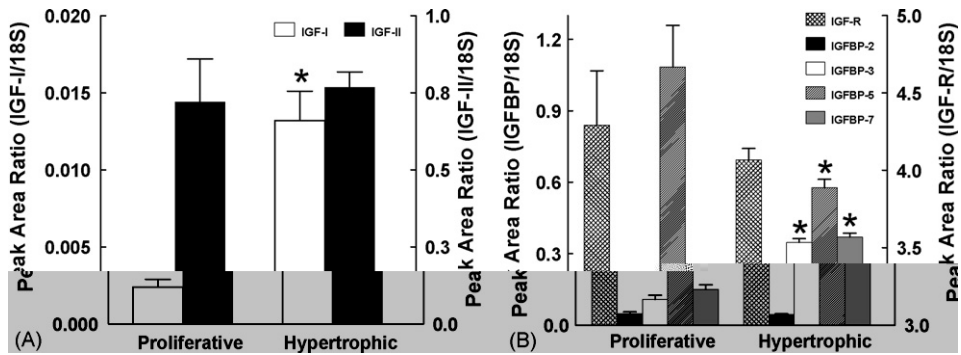


Fig. 1. Expression of the (A) IGF-I and IGF-II and (B) type 1 IGF receptor (IGF-R) and IGF-binding protein (IGFBP) genes in freshly isolated proliferative and hypertrophic chondrocytes. RT-PCR was used to quantify the level of expression relative to an 18S rRNA internal standard. Values are reported as means \pm S.E.M. ($n = 3$). Asterisk denotes statistically significant ($P < 0.05$) differences between proliferative and hypertrophic chondrocytes.

of IGF-I was low, there was a significant increase (5.5-fold) in expression in hypertrophic as compared to proliferative chondrocytes (Fig. 1A). The opposite result was found for IGFBP-5 where there was a significant decrease (1.9-fold) associated with hypertrophy (Fig. 1B). However, significant increases in IGFBP-3 (3.2-fold) and IGFBP-7 (2.5-fold) were observed in hypertrophic as compared to proliferative chondrocytes (Fig. 1B).

3.2. Influence of insulin-like growth factors (IGFs) on chondrocyte proteoglycan synthesis

In a preliminary study, IGF-I and IGF-II were observed to have equal ability to stimulate S^{35} incorporation by cultured chondrocytes. The results of repeating this experiment with a wider range of growth factor concentrations are shown in Fig. 2. Both IGF-I and IGF-II stimulated S^{35} incorporation in a dose-dependent manner. Although IGF-II resulted in numerically superior values, the results were not statistically different.

3.3. Influence of insulin-like growth factors on markers of chondrocyte differentiation

The influence of IGFs on chondrocyte differentiation was investigated using the serum-free culture system described by Rosselot et al. [22] with modifications by Praul et al. [21]. In this system, proliferative zone chondrocytes multiply and then hypertrophy in 48 h when appropriately stimulated. This closely approximates the progression of events in vivo. The data illustrating the expression of *Ihh*, as well as types X and II collagen are shown in Fig. 3. The results show that IGF-I stimulates the expression of type X collagen and *Ihh* in a time-dependent manner. During the same period, there is a decline in the expression of type II collagen which is associated with the proliferative zone [20]. The *Ihh* cDNA hybridizes with two mRNA transcripts (3.1 and 1.9 kb) under stringent conditions. The pattern for each was identical. In a preliminary experiment, IGF-I and IGF-II were compared using the design shown in Fig. 3. The efficacy of the two factors was equal (data not shown).

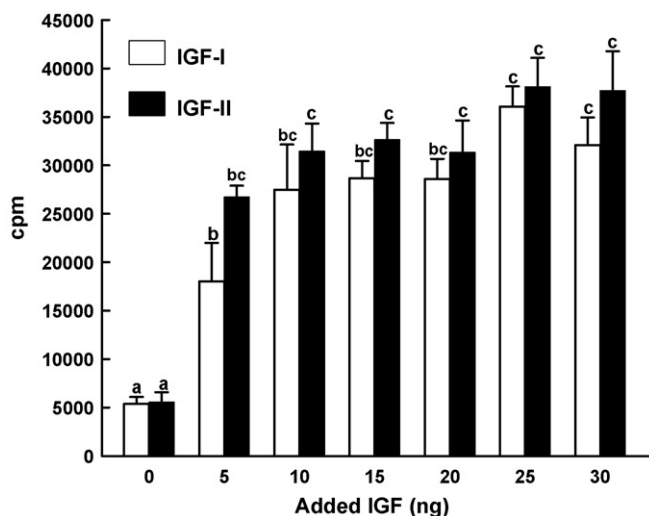


Fig. 2. Effect of IGF-I and IGF-II on $^{35}\text{SO}_4$ incorporation by cultured growth plate chondrocytes. Sulfate incorporation was determined by pulse labeling the cells with $^{35}\text{SO}_4$ 12 h prior to harvesting. Labeling was performed on four wells per treatment with cells obtained from approximately 10 chicks. The results shown are those combined from two separate experiments. Values are reported as means \pm S.E.M. Different letters above each bar denote statistically significant ($P < 0.05$) differences for mean comparisons.

However, since this was a single experiment, the data could not be subjected to statistical analysis.

4. Discussion

Our data indicate that IGF-II is the predominant IGF peptide expressed in growth plate tissue of the post-natal chick. This observation is consistent with the observation of Bautista et al. [6] who found IGF-II to be present in higher quantities than IGF-I in extracts of skeletal tissue from 10 different vertebrates. Other reports by Shinar et al. [7] and Wang et al. [8] suggest that IGF-II plays a major role in post-natal skeletal metabolism. In a more recent publication, Wang et al. [2] used mice with genetic deletions of IGF-I or growth hormone receptor to study longitudinal bone growth. While the observations supported a dual role (IGF-I-dependent, IGF-I-independent) for GH in longitudinal bone growth, it was concluded that IGF-II may mediate some of the effects of GH on the growth plate.

The observation that IGF-II may play an important role in post-natal skeletal metabolism is often overlooked since most of the literature focuses upon IGF-I. Questions being addressed include the IGF-I-independent and IGF-I-dependent roles for GH stimulation of longitudinal bone growth [2], determining the relative importance of endocrine versus autocrine/paracrine IGF-I in regulation of body growth [27] and the continued investigation of the role of IGF-I in the acquisition and maintenance of bone mass [28].

Our results show that IGF-I and IGF-II have equivalent *in vitro* activity for epiphyseal growth plate chondrocyte matrix synthesis. This is not surprising since, in birds, both pep-

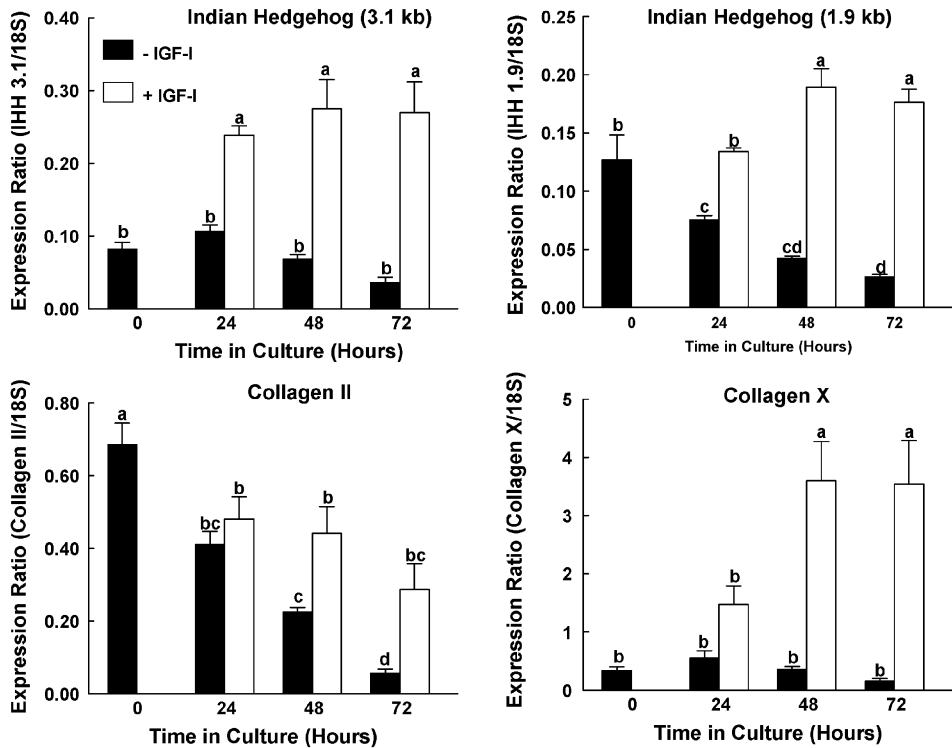


Fig. 3. Results of Northern blotting for Indian hedgehog (3.1 and 1.9 kb transcripts), collagen X and collagen II. Total mRNA was obtained from freshly isolated proliferative cells as well as chondrocytes cultured in either modified Webbers media (MWM) without added IGF-I (–IGF-I) or MWM with 25 ng/ml IGF-I (+IGF-I) for 24, 48 or 72 h. Values are reported as means \pm S.E.M. ($n=3$). Different letters above each bar denote statistically significant ($P<0.05$) differences for mean comparisons within each gene.

tides are known to act through the IGF-I receptor [29]. Furthermore, immunohistochemical studies show that these peptides are localized in the pre-hypertrophic and hypertrophic zones of this tissue, the site of chondrocyte differentiation [10–12]. In these studies, anti-human IGF-I was used to localize IGF peptides. We used dot blotting [12] to confirm the report of Ralphs et al. [9] that this antibody cross-reacts with both avian IGF-I and IGF-II.

Although there is extensive literature describing both proliferative and differentiation roles for IGFs in growth plate physiology, our data favor a role in differentiation. We have observed a stimulation of type X collagen and Ihh expression by the addition of IGF-I to this serum-free culture system. This is consistent with our previous report that IGF was a poor mitogen for proliferative zone chondrocytes even in the presence of other growth factors or serum [16].

The bioactivity of IGF-I and IGF-II, both in circulation and in tissues, is closely regulated by a series of high affinity IGF-BPs that control tissue IGF availability and binding of IGFs to their cognate IGF-Rs by associating with molecules in the extra-cellular matrix or by binding directly to the cell surface [30]. IGF-BPs exert both stimulatory and inhibitory

actions with respect to IGF bioactivity, but they can also produce IGF-independent actions as well [31]. To date, six distinct IGBPs have been identified and characterized in a number of different vertebrate species. Also, a number of low affinity IGFBP-related proteins (IGFBP-rP1–10) including IGFBP-7 (also known as IGFBP-rP1 or mac25) have been reported [32]. Although IGFBPs circulate throughout the body in the bloodstream, regulation of IGFBP expression within the local tissue environment has been suggested to be an important factor in modulating IGF action on growth plate chondrocytes [33–35]. With the exception of IGFBP-1, all have been reported to be expressed locally in mammalian growth plate chondrocytes [8,36–38]. All six of the IGFBP peptides have been reported to be associated with growth plate chondrocytes based on immunohistochemical analysis, suggesting that they may be available from general circulation as well [33]. Exogenous IGFBP-2 was shown to potentiate IGF-II mitogenic effects on fetal ovine growth plate chondrocytes [33]. IGFBP-1, -2, -4 and -6 inhibited IGF-I-stimulated proliferation, whereas IGFBP-3 and -5 enhanced this process in cultured growth plate chondrocytes [34,35].

We found that IGFBP-1 and -4 were not expressed in proliferative or hypertrophic avian chondrocytes. Since no IGFBP-6 gene homologue has been identified for chickens, presumably this binding protein is also not expressed. The high level of expression of IGFBP-5 detected in proliferative chondrocytes in this study agrees with the findings of Kiepe et al. [37] who reported that IGF-I-stimulated cell proliferation up-regulated expression of IGFBP-5 in cultured growth plate chondrocytes. However, the significant down-regulation we observed in IGFBP-5 in hypertrophic chondrocytes differs from the marked up-regulation reported for rat chondrocytes during the process of differentiation [38]. Perhaps this discrepancy reflects species differences in the regulation of IGFBP-5 gene expression. It has recently been reported that IGFBP-5 is regulated by the sonic hedgehog signaling pathway during chicken embryogenesis [39], whereas Kiepe et al. [37] reported that IGF-I-induced IGFBP-5 gene expression in rat chondrocytes was mediated through the phosphatidylinositol-3 kinase signaling pathway. Regulation of mammalian chondrocyte IGFBP-3 gene expression has been reported to be complex, exhibiting a bi-phasic pattern during cellular differentiation [37]. The up-regulation of both IGFBP-3 and -7 observed in hypertrophic as compared to proliferative chondrocytes in this study is consistent with a potential role for these particular binding proteins in mediating aspects of cellular differentiation. Finally, Kiepe et al. [38] found that IGF-I expression increased 2-fold during the process of differentiation in cultured growth plate chondrocytes which is similar to our observation of a 5.5-fold up-regulation in IGF-I expression in hypertrophic as compared to proliferative chondrocytes. However, consistent with our findings, IGF-II was reported to be the predominant IGF expressed by proliferating and differentiating mammalian chondrocytes [8,33].

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